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Validation of Egg Yolk Antibody Testing as a Method to Determine Influenza Status in White Leghorn Hens

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SUMMARY. Determination of the avian influenza (AI) status of a flock has traditionally been done by detection of serum antibodies. However, for many diseases, detection of antibodies in egg yolk has been effective in monitoring the disease status of laying flocks. This study compared the utility of egg yolk *vs.* serum for determining AI status in laying hen flocks. Specific-pathogen-free white leghorn hens were inoculated via the respiratory tract with a low-pathogenic H7N2 AI virus or sterile allantoic fluid or subcutaneously with an inactivated oil emulsion vaccine produced from the same AI virus or normal allantoic fluid. Antibody levels were determined by the agar gel immunodiffusion (AGID) test, the hemagglutination-inhibition (HI) test, and the enzyme-linked immunosorbent assay (ELISA). Anti-influenza antibodies were detected in sera of all live virus–inoculated hens by day 7 postinoculation (PI) (AGID and ELISA tests), but detection of antibodies in egg yolk was delayed by a few days, with all being positive by day 14 PI. Sera from all vaccinated hens were positive by day 14 PI (AGID and ELISA tests), and egg yolk was positive by day 18 PI. The HI test was less sensitive than the ELISA and AGID tests in detecting anti-influenza antibodies in both sera and yolk. Serum and yolk from all control birds remained negative throughout the study. These studies show that currently used serologic tests can detect antibodies in serum and yolk samples from hens exposed to live AI virus or from those that have been vaccinated. Antibody is detected earlier in the serum than in the yolk and antibody is detected earlier from birds exposed to a live infection compared to birds vaccinated with an inactivated oil emulsion vaccine.

RESUMEN. Validación de una prueba de medición de anticuerpos en yema de huevo como un método para determinar el estado de influenza aviar en gallinas leghorn blancas.

La determinación del estado de influenza aviar en una parvada se ha realizado tradicionalmente mediante la detección de anticuerpos en el suero. Sin embargo, para muchas enfermedades, la detección de anticuerpos en la yema de huevo ha sido efectiva para conocer el estado de una enfermedad en una parvada de postura. Este estudio se comparó la utilidad de la yema de huevo en comparación con el suero para determinar el estado de influenza aviar en parvadas de postura. Gallinas de postura Leghorn blancas libres de patógenos específicos fueron inoculadas por el tracto respiratorio con un virus de influenza H7N2 de baja patogenicidad o con fluido alantoideo estéril o con una vacuna inactivada emulsionada en aceite producida a partir del mismo virus. Los niveles de anticuerpos fueron determinados por la prueba de inmunodifusión en agar, por la inhibición de la hemoaglutinación y por el ensayo de inmunoabsorción ligado a enzimas (de las siglas en Inglés ELISA). Los anticuerpos anti-influenza fueron detectados en el suero de todas las gallinas inoculadas con virus vivo al día 7 post-inoculación mediante inmunodifusión en agar y ELISA, pero la detección en yema de huevo se retrasó algunos días mostrándose todas las aves positivas al día 14 postinoculación. El suero de todas las gallinas vacunadas fue positivo al día 14 postinoculación mediante la inmunodifusión en agar y la prueba ELISA y se detectaron positivos en yema al día 18 postinoculación. La prueba de inhibición de la hemoaglutinación fue menos sensible que ELISA e inmunodifusión en agar para detectar anticuerpos contra influenza tanto en suero como en yema de huevo. El suero y la yema de huevo de todas las aves control permanecieron negativos durante el estudio. Estos estudios muestran

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que las pruebas serológicas que se usan actualmente pueden detectar anticuerpos en muestras de suero y de yema de huevo de gallinas expuestas a virus vivo de influenza aviar o de aquellas que han sido vacunadas. La presencia de anticuerpos se detecta más temprano en el suero que en la yema de huevo y en forma más temprana en aves expuestas a la infección en comparación con las aves vacunadas con una vacuna inactivada emulsionada en aceite.

Key words: antibody, avian influenza, egg yolk, poultry, serum

Abbreviations: AGID = agar gel immunodiffusion; AI—avian influenza; ELD_{50} = 50% embryo lethal dose; ELISA = enzyme-linked immunosorbent assay; HI = hemagglutination-inhibition; PBS = phosphate-buffered saline; PI = postinoculation; SPF = specific pathogen free

Current avian influenza (AI) surveillance programs require the testing of serum from poultry flocks to obtain an official status as "AI-free." Furthermore, importation of poultry or poultry products requires the exporting country to certify that the flock is free of AI by testing serum for anti-influenza antibodies. In contrast, with many poultry diseases, diagnosis and disease monitoring can be accomplished by evaluating samples, as is the case with egg yolk testing, which requires less labor to obtain samples and which is less intrusive for the bird. The purpose of this study is to determine whether testing of egg yolk for anti-influenza antibodies would be as reliable as testing serum.

MATERIALS AND METHODS

Experiment 1: Live virus inoculation. A group of 20 regularly laying 30-wk-old specific-pathogen-free (SPF) white leghorn hens was housed such that eggs could be collected and identified by bird number. Twelve hens were inoculated by the intranasal and intratracheal routes (half volume by each route) with 10^7 50% embryo lethal dose (ELD_{50}) of a recent H7N2 low-pathogenic AI virus isolate, A/chicken/PA/9701524/98. Eight hens were similarly inoculated with diluted normal allantoic fluid to serve as controls. Eggs were collected daily and labeled by hen number and days postinoculation (PI). Hens were wing-bled on days 0, 7, 14, 18, and 21 PI. Yolk from individual eggs collected on those days was harvested and mixed. Serum and yolk samples were tested by agar gel immunodiffusion (AGID) test, the hemagglutination-inhibition (HI) test, and the enzyme-linked immunosorbent assay (ELISA) for antibodies against influenza antigens.

Experiment 2: Inactivated vaccine inoculation. A group of 20 regularly laying 39-wk-old SPF white leghorn hens was housed in such a way that eggs could be collected and identified by bird number. Twelve hens were inoculated subcutaneously with 0.2 ml of an inactivated oil emulsion vaccine produced using the A/chicken/PA/9701524/98 H7N2 virus. Eight hens were inoculated subcutaneously with an oil emulsion vaccine using normal allantoic fluid as antigen to serve as controls. Eggs were collected daily and labeled by hen number and days PI. Hens were wing-bled on days 0, 7, 14, 18, and 21 PI. Yolk from individual eggs collected on those days was harvested. Serum and yolk samples were tested by AGID, HI, and ELISA tests for anti-influenza antibodies.

Serologic testing. AGID tests were run as previously described (5). AGID antigen was produced from infected chorioallantoic membranes of chicken embryos, as described by Beard (1). Yolk samples were run in duplicate using full-strength as well as a 1:1 phosphate-buffered saline (PBS) extraction. The HI test was a standard beta test using four hemagglutinating units of antigen prepared from chicken embryos inoculated with A/turkey/Oregon/71 (H7N3) virus (3). ELISA tests were conducted using a commercial AI ELISA test-kit (IDEXX, Westbrook, ME).

RESULTS

Experiment 1. All serum samples (12 of 12) from hens inoculated with the live virus were positive on day 7 PI in the AGID and ELISA tests, and 50% of samples were positive by the HI test (Table 1). Serum samples were positive in all three tests by day 14 PI. Yolk from eggs laid by the hens on day 7 PI were negative in all three tests and were positive in all tests on day 14 PI. Serum and yolk from all control birds remained negative throughout the study. There was no difference in the AGID test results between using full-strength yolk or yolk extracted 1:1 with PBS.

Experiment 2. Serum from the 12 vaccinated hens was negative on day 7 PI in the AGID and HI tests, but 42% (5/12) were positive in the ELISA test (Table 2). All 12 serum samples were positive by day 14 PI in the AGID and ELISA tests and by day 18 PI using the HI test. Six of 12 (50%) and 8 of 12 (67%) yolk samples were positive on day 14 PI in the AGID and ELISA tests, respectively, and all samples were positive by day 18 PI. All yolk samples were negative in the HI test up to day 14 PI. By day 18 PI, 42%

Table 1. Antibody response in serum and yolk collected from hens inoculated topically by the intranasal and intratracheal routes with 10^7 ELD_{50} of live A/Ck/PA/9701524/98 (H7N2) virus.

Days PI	Antibody source	Serology (No. positive/total)[A]		
		AGID	HI	ELISA
0	Serum	0/12	0/12	0/12
	Yolk	0/12	0/12	0/12
7	Serum	12/12	6/12	12/12
	Yolk	0/8	0/8	0/8
14	Serum	12/12	12/12	12/12
	Yolk	8/8	8/8	8/8
18	Serum	12/12	12/12	12/12
	Yolk	10/10	10/10	10/10
21	Serum	12/12	12/12	12/12
	Yolk	10/10	10/10	10/10

[A]AGID = agar gel immunodiffusion test; HI = hemagglutination-inhibition test; ELISA = enzyme-linked immunosorbent assay.

Table 2. Antibody response in serum and yolk collected from hens inoculated subcutaneously with 0.2 ml oil emulsion vaccine produced with A/Ck/PA/9701524/98 (H7N2) virus.

Days PI	Antibody source	Serology (No. positive/total)[A]		
		AGID	HI	ELISA
0	Serum	0/12	0/12	0/12
	Yolk	0/12	0/12	0/12
7	Serum	0/12	0/12	5/12
	Yolk	0/12	0/12	0/12
14	Serum	12/12	10/12	12/12
	Yolk	6/12	0/12	8/12
18	Serum	12/12	12/12	12/12
	Yolk	12/12	5/12	12/12
21	Serum	12/12	11/12	12/12
	Yolk	12/12	10/12	12/12

[A]AGID = agar gel immunodiffusion test; HI = hemagglutination-inhibition test; ELISA = enzyme-linked immunosorbent assay.

(5/12) were positive, and by day 21 PI, 83% (10/12) were positive. Serum and yolk from all control birds remained negative throughout the study.

DISCUSSION

The absence of antibodies against influenza antigens has been used in surveillance and certification programs to assure the absence of influenza infection in poultry flocks (2,5). Catching and bleeding of poultry for serum collection has been routine in many meat poultry flocks and has yielded sera samples used to monitor for evidence of various diseases and effectiveness of immunization programs. For laying flocks, an abundant alternative sample type, the egg, is a rich source of antibody and has been used for serologic monitoring of laying flocks against various poultry diseases, including Newcastle disease. During the 1983–84 H5N2 AI outbreak in the northeastern United States, egg yolk extraction procedures were developed and used to determine influenza status of layer flocks (4). However, egg yolk was not validated against sera samples as an acceptable alternative for serologic testing.

In the current time course study, sera and egg yolk samples from laying hens were compared by AGID, HI, and ELISA tests for detecting antibodies against influenza antigens. Anti-AI viral antibodies were detected in the serum and yolk of hens exposed to live AI virus or from those that have been previously vaccinated with inactivated whole AI virus. All three serologic techniques detected antibodies in sera and yolk of AI virus–inoculated or –vaccinated hens, but the earliest detection of antibodies was through use of the ELISA test, followed by the AGID test, with the HI test being "last" in terms of detection of anti-influenza antibodies. The serologic tests detect antibody earlier in the serum than in the yolk samples and earlier from chickens inoculated with the live AI virus than in vaccinated birds. However, on day 14 PI or later, yolks were as effective as sera samples for detecting anti-influenza antibodies in virus-inoculated hens. Similar results were seen on day 18 or day 21 for yolk *vs.* serum from vaccinated hens. Egg yolk can be used as an alternative sample to serum for detecting anti-influenza antibodies in a surveillance program.

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